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DRAFT GUIDANCE DOCUMENT ON THE APPLICATION OF HISTOPATHOLOGY TO OCULAR TEST METHODS

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INTRODUCTION

1. This Guidance Document, which includes detailed procedures for collecting and using histopathology data on ocular tissues, accompanies the OECD Test Guidelines for the Bovine Corneal Opacity and Permeability (BCOP) test method and the Isolated Chicken Eye (ICE) test method (1)(2). The BCOP and ICE test methods have been accepted by U.S. and European regulatory agencies for use under certain circumstances and with specific limitations to identify ocular corrosives and severe irritants[*] (i.e., U.S. Environmental Protection Agency [EPA] Category 1, EU R41, the United Nations [UN], and Globally Harmonized System of Classification and Labeling of Chemicals [GHS] Category 1)[**]. While these tests are not considered valid as complete replacements for the *in vivo* rabbit eye test, the BCOP and ICE test methods (6)(7) are recommended as part of a tiered testing strategy for regulatory classification and labeling within a specific applicability domain (8)(9). Substances that are positive in these assays and considered ocular corrosives or severe irritants after a weight-of-evidence decision need not be tested in animals. A substance that tests negative would need to be tested with a valid and accepted test method (e.g., OECD TG 405 [10]) that can correctly identify possible false negatives as corrosives or severe irritants, or as irritants that induce reversible ocular damage that resolves within 21 days.

2. Histopathology data have not been formally evaluated for use in the BCOP or ICE test methods (6)(7). However, a histopathological assessment can be included on a case-by-case basis to help classify test substances as corrosives/severe irritants (8). Histopathology may be useful when a more complete characterization of damage is needed. Users are encouraged to preserve tissues and prepare histopathology specimens that can be used to develop a database and decision criteria that may further improve the accuracy of these test methods.

3. A histopathological assessment may also be used to support the development of other alternative ocular toxicity test methods (e.g., Isolated Rabbit Eye test method [11], Porcine Corneal Opacity and Permeability Assay [12], 3-dimensional human corneal tissue constructs).

4. Histopathology may be used as an additional endpoint in the *in vivo* rabbit eye test to more thoroughly evaluate ocular damage, as well as to provide a reference against

[*] For the purpose of this Guidance Document, *severe irritants* are defined as those that induce ocular lesions that persist in the rabbit for at least 21 days after administration of the test substance.

[**] EPA –Category I: Corrosive (irreversible destruction of ocular tissue) or corneal involvement or irritation persisting for more than 21 days (3)
EU – R41: Production of tissue damage in the eye, or serious physical decay of vision, following application of a test substance to the anterior surface of the eye, which is not fully reversible within 21 days of application (4)
GHS Category 1: Production of tissue damage in the eye, or serious physical decay of vision, following application of a test substance to the anterior surface of the eye, which is not fully reversible within 21 days of application (5)

which to compare effects induced *in vitro*. These additional data may help in development of a more accurate, mechanistically based alternative to the *in vivo* rabbit eye test.

5. The purpose of this Guidance Document is to describe the procedures for the collection, preservation, and preparation of ocular tissues for use in collecting histopathology data. This document does not provide guidance on the interpretation of histopathology data or the associated decision criteria to be used for ocular hazard classification. When it becomes available, relevant information will be added to this Guidance Document.

6. Definitions are provided in **Annex 1**.

INITIAL CONSIDERATIONS AND LIMITATIONS

7. Histopathology data have not been formally evaluated for use in any *in vitro* ocular assay. Histopathology is potentially useful for chemical classes or formulations that are not well characterized in these assays, where the mode of action cannot be easily predicted, when delayed effects might be anticipated, or when a more complete characterization of damage is needed (13)(14). Therefore, users are encouraged to preserve tissues for histopathology analysis.

8. A limitation of histopathology is that, although many of the ocular effects seen *in vivo* (and, to some degree, their severity) can be further evaluated based on the types of lesions observed histologically, these may not account for all of the effects assessed *in vivo.* For example, the reversibility of corneal lesions cannot be evaluated by histopathology, but an assessment of the initial depth of corneal injury has been proposed to predict irreversible or reversible effects (13).

9. All procedures using animal eyes should follow the institution's applicable regulations and procedures for handling animal substances that include but are not limited to tissues and tissue fluids. Universal laboratory safety precautions are recommended.

10. To facilitate consideration of histopathology as a useful endpoint for *in vitro* and *in vivo* ocular toxicity testing, users are encouraged to contact the international validation organizations (i.e., the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods [NICEATM], the European Centre for the Validation of Alternative Methods [ECVAM], or the Japanese Center for the Validation of Alternative Methods [JaCVAM]) when tissues and data are available.

PRINCIPLE OF THE PROCEDURE

11. Enucleated, transected globes or isolated portions of the anterior segment (e.g., the cornea) are trimmed, fixed, dehydrated, and infiltrated with paraffin wax. Paraffin-embedded tissue is sectioned with a microtome to obtain cross-sections of the tissues of

interest, which are placed on microscope slides. Slides are then incubated in a common histological stain that provides adequate contrast of the cells included in the tissue section (e.g., hematoxylin and eosin). A pathologist or a trained technician reviews the stained slides to determine the location, depth, and type of injury. Once unbiased observations are recorded, the relationship of the test substance in terms of its chemical class (e.g., bleach, oxidizer, ionic or nonionic surfactant, solvent, acid, or base), physical characteristics, and other parameters to the production of irreversible or reversible injury can be considered and ascertained.

12. Histopathology should be considered as an additional endpoint for currently used ocular toxicity test methods. It may also help develop or optimize new *in vitro* alternative ocular toxicity test methods.

13. It is very important that the full thickness of corneal tissue be available for analysis (e.g., epithelium, stroma, endothelium, and Descemet's membrane). For example, evaluations are typically conducted from the top epithelial layer down through the stroma and into the endothelial layer. Since test substances are applied topically, this permits initial assessment of any damage to the nonkeratinized basal, wing, or superficial epithelial cells (e.g., keratinization, ulceration, surface calcification); stromal damage close to Bowman's layer; disruption of stromal collagen or keratocytes (e.g., thinning, thickening, scarring, neovascularization, apoptosis); or damage to the endothelium (i.e., swelling, destruction, changes in morphology) or Descemet's membrane (14).

PROCEDURE

Source of Tissue for Histological Analysis

14. The source of tissue to be considered for histological analysis includes whole eyes or isolated portions of the anterior segment (e.g., cornea) obtained at the end of an ocular toxicity test (*in vitro* or *in vivo*). All information related to the type and treatment of a particular tissue sample should be included in the **Test Report** (see paragraph 40).

Sample Identification

15. Each sample should be assigned a unique identifier that will allow it to be traced back to the study from which it was obtained (14)(15).

Tissue Preparation

16. Methods for enucleation of the eye may vary slightly for different species. Prior to enucleation, it is important to note any gross findings or changes in the cornea (e.g., neovascularization, changes in color or pigmentation, opacity, swelling, presence of particulate matter, or other corneal defects) in a treated eye relative to adjacent normal ocular tissue. The globe should be dissected free from surrounding tissue by deflecting the eyelids and/or nictitating membrane to permit access to the conjuctivae, ocular muscles, blood vessels and glands (see Appendices A1 in [6][7] and D and E in [8]). These tissues are cut using angled forceps and curved scissors to allow the globe to be

lifted from the orbit with gentle pressure. The eyes may be perfused with physiological saline or buffer to prevent drying (see Appendices A1 in [6][7] and D and E in [8]). Enucleated globes may also be kept hydrated by covering the tissue with fixative-soaked gauze or cloth (16).

17. Prior to fixation, scissors should be used to trim enucleated eyes of excess fat and/or connective tissue. To maintain intraocular pressure, preservation of a visual length of the optic nerve is recommended. Where applicable, isolated corneas (e.g., for the BCOP assay) should be trimmed of excess scleral tissue (see Appendices A1 in [6][7] and D and E in [8]). Typically, a 2- to 3-mm rim of scleral tissue is preserved to allow tissue to be manipulated without damaging the corneal surface. Corneas from isolated eyes are obtained by sectioning the anterior portion of the eye.

Tissue Preservation

18. Tissue fixatives prevent autolysis by inactivating autolytic enzymes that are released postmortem (17). Fixation also hardens the tissue thereby allowing thin sections to be cut without inducing mechanical artifacts (e.g., compression of the tissue). Factors that affect tissue fixation include time and temperature during incubation, the volume of the fixative relative to tissue size, the physicochemical properties of the fixative, and the concentration of the fixative (17)(18).

19. To prevent the tissues from drying out, which would induce substantial artifacts, they must remain immersed in fixative before processing and embedding.

20. Tissues should be placed in pre-labeled containers (see paragraph 15) filled with enough fixative (see paragraph 22) to completely cover the tissue. Smaller tissues may be placed into cassettes; however, for consistency in sectioning, care should be taken to orient them so that the epithelial (anterior) surface faces the top of the cassette (14).

21. Most histology protocols recommend a volume at least 5- to 10-fold greater than the size of the tissue (16)(19), although Banks (17) recommends up to a 30-fold fixative-to-tissue size ratio.

22. The depth of penetration of most fixatives is directly proportional to the square root of the duration of fixation (t) dependent on the coefficient of diffusibility (k) of the fixative, which averages to 1 for typically used fixatives. Fixation time thus translates to the square of the distance the fixative must penetrate. At a rate of 1 mm/hour, the time of fixation for a 10-mm sphere in neutral buffered formalin (NBF) will be $(5)^2$ or 25 hours of fixation (20). Therefore, tissues are typically fixed for at least 24 hours at room temperature. However, the reported range for fixation is 4 to 48 hours (21)(22), and some protocols perform fixation at 4°C (21)(23).

23. The fixatives most commonly used for ocular tissues are 10% NBF and Davidson's Fixative (see **Annex II**). Other fixatives that have been used for ocular tissues include 4% glutaraldehyde (24), 4% formalin (Menk Prinsen, Netherlands Organisation for Applied Scientific Research, personal communication), a mixture of 2.5% glutaraldehyde and 2% formaldehyde (25)(22), and 4% paraformaldehyde (21)(23).

Post-fixation Tissue Trimming

24. Prior to initiating the tissue-processing step, it may be necessary to trim the fixed tissues to ensure that they are adequately dehydrated and infiltrated with paraffin wax (see paragraph 24). Any post-fixation trimming should be done with a sharpened scalpel or razor blade to minimize tissue artifacts.

Tissue Processing and Embedding

25. Tissues contain approximately 75% water (17) and must be thoroughly dehydrated prior to embedding. Other water-miscible solvents have been reported to remove water (e.g., n- butanol, dioxane, isopropanol, propanol, tetrahydrofuran, and tetrahydrofurfuryl alcohol [17][18][26][27]), but tissue is most commonly dehydrated by immersing in a graded (i.e., increasing concentrations) alcohol series such as ethanol from 60%-70%, 90%-95%, and 100% (15)(19). Lower concentrations, such as 30% ethanol, are recommended for delicate tissue (15).

26. A routine schedule for processing eyes with a tissue processor is provided in Barequet et al (29). Enucleated globes that are initially fixed overnight in 10% NBF are dehydrated in 4% phenol/70% alcohol for 1 hr each. Phenol is added to soften the sclera and lens. The eyes are then incubated in two separate stations of 95% alcohol (1 hr each), followed by two separate stations of 100% alcohol (1.5 hr each). Tissue-clearing steps include incubations in 50% alcohol/50% chloroform for 2 hr, followed by two separate stations of 100% chloroform (2 hr/each). Xylene may be used instead of chloroform. Tissue is then infiltrated with liquid paraffin in two separate 2-hr incubations. This schedule may require modification depending on the manufacturer's specifications and the type of tissue processor used.

27. Because alcohols are not miscible with the paraffin wax used for embedding, a substance that is miscible with ethanol and paraffin wax in the absence of water must be used for intermediate clearing. This step also increases the transparency of the resulting tissue section (i.e., "tissue clearing" [28]). Xylene is the most common clearing agent used , although others have been used, including benzene, chloroform, n-butanol, n-butyl acetate, amyl acetate, ligroin, petroleum solvents (mainly hexanes), toluene, and trichloroethane, or terpenes such as cedarwood oil, limonene, and terpineol (17)(18)(26)(27). Many of these solvents may be toxic or potentially carcinogenic, so it is important to consult the Material Safety Data Sheets to determine proper handling conditions prior to use.

28. Because of the damage and resulting morphological artifacts produced from elevated temperatures, tissue should be dehydrated and cleared at room temperature.

29. To allow sections to be cut on a microtome and applied to glass microscope slides (see paragraph 33), ocular tissue is routinely embedded in paraffin wax, a polycrystalline mixture of solid hydrocarbons (14)(23)(24)(29)(30).

30. Plastic materials such as glycol methacrylate have been used to embed corneal or globe tissue of the rabbit (22). Plastic embedding has some advantages over paraffin embedding for corneal disc preparations (e.g., no heat exposure, reduced distortion). However, in the absence of published reports favoring plastic embedding materials over paraffin wax for ocular histopathology, this Guidance Document does not focus on them.

31. Processed tissues should be embedded so as to maintain the appropriate orientation in the hardened tissue block once the paraffin cools. For example, true corneal cross-sections (i.e., anterior to posterior) are usually desired to permit an accurate measurement of the corneal thickness due to swelling caused by the test substance relative to the negative control. Therefore, the tissue must be embedded in the block on its edge in the correct orientation to permit such sections. For whole eyes (i.e., bisected or transected globes), users should follow the individual standard operating procedures and ensure that the optimal orientation is used to visualize the tissue of interest.

Tissue Sectioning and Slide Preparation

32. Once embedded, the tissue is usually sectioned using a microtome with a sharpened steel blade. Depending on the type of microtome used, the thickness of microtome sections for tissue is generally 3-8 μm (15)(17)(26)(28), which is the range of thickness suggested by Lee (31). The microtome should be placed on a stable surface composed of a dense material that will minimize vibrations (e.g., a marble desktop). Vibrations of the knife blade could cause substantial tissue artifacts (15).

33. For corneas, ribbons of tissue sections (i.e., a series of tissue sections in which the trailing edge of one section adheres to the trailing edge of the next section) are usually floated on warm water to reduce wrinkles when they are mounted on glass slides (14)(17)(18). However, it is important to remove tissue from the water before it expands and causes artifactual spaces between tissues, cells, and extracellular fibers (15)(28). There is no standardized length of time for allowing the tissue to float, but expansion is easily visible as the tissue separates from the paraffin wax.

34. Poly-L-lysine-coated glass microscope slides are often used to ensure that the tissue sections adhere to the microscope slide throughout the staining procedures,. Alternatively, gelatin can be added to the water bath (15).

35. Sharp knife blades should always be used; dull blades can cause microtome artifacts such as compression lines or chatter, knife marks or tears, and/or uneven thickness of the tissue section (15)(28).

Staining of the Tissues

36. For routine histopathological evaluations, such as that addressed in this Guidance Document, tissues are most commonly stained with hematoxylin and eosin (H&E) (32). The basic dye hematoxylin colors basophilic structures (e.g., those containing nucleic acids) and acidic components with a blue-purple hue, while the alcohol-based acidic eosin Y, colors eosinophilic structures (those composed of intracellular or extracellular protein) or basic cell components such as cytoplasm bright pink. Thus cell nuclei appear blue-purple, while the cytoplasm is pink (17)(32). See **Annex II** for stain recipes and **Annex III** for a sample H&E staining protocol.

37. Adequate contrast is essential to an accurate evaluation. Therefore, the recommended stain recipes and incubation times and temperatures should be closely followed to prevent stain color-intensity changes or variations within or between studies. Poorly stained slides should be re-stained and/or re-sectioned.

Evaluation of Quality and Acceptability of the Corneal Sections

38 Tissues from animals/samples treated with test substance should be processed together with positive and negative control tissues. Negative control tissues may be used to determine acceptability of the other slides in a group. They may also be used to evaluate the quality of the stain, artifacts, tissue architecture, and tissue thickness (14). Positive controls may be used to develop a historical database for ocular damage produced by severe irritants. Benchmark controls could be used to identify potential mechanisms of action based on the type of injury produced by a given chemical or product class (e.g., oxidizer, surfactant).

DATA AND REPORTING

Preparation of Digital Images

39. Digital images of all tissue sections should be prepared in order to provide a permanent record of the degree of damage at the indicated depth observed in the treatment group relative to that in the negative and positive control groups. Although the slides can be archived and retrieved for repeated evaluations, histological stains fade over time, complicating future evaluations.

Test Report

40. The test report should include the following information, if relevant to the conduct of the study:

Test and Control Substances

- Chemical name(s) such as the structural name used by the Chemical Abstracts Service (CAS), followed by other names, if known
- The CAS Registry Number (RN), if known
- Purity and composition of the substance or preparation (in percentage[s] by weight), to the extent this information is available
- Physicochemical properties such as physical state, volatility, pH, stability, chemical class, water solubility relevant to the conduct of the study
- Treatment of the test/control substances prior to testing, if applicable (e.g., warming, grinding)
- Stability, if known

Histology Report

- Unique sample identifier
- Type of tissue analyzed (e.g., cornea, whole eye)
- Tissue species (e.g., bovine, rabbit)
- Number of tissues analyzed for each test substance and control (e.g., n=3)
- Fixative, dehydration and clarifying agents, and protocols used
- Embedding material, infiltration solvents, and concentrations used
- Thickness of tissue sections
- Stain and the associated staining protocol used
- Information on instruments used

Information About the Sponsor and the Test Facility

- Name and address of the sponsor
- Name and address of the test facility
- Name and address of the Study Director
- Storage and transport conditions of eyes (e.g., date and time of eye collection, time interval prior to testing)

Results

- Digital images

Quality Assurance Statement for Good Laboratory Practice (GLP)-compliant Studies

- This signed statement lists the types and dates of inspections during the study and the dates any inspection results were reported to the Study Director. It also confirms that the final report reflects the raw data.

If GLP-compliant studies are performed, then additional reporting requirements set forth in the relevant guidelines (34)(35)(36) should be followed.

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ANNEX I

DEFINITIONS

Benchmark control: A sample containing all components of a test system and treated with a known substance (i.e., the benchmark substance) to induce a known response. The sample is processed with test substance-treated and other control samples to compare the response produced by the test substance to the benchmark substance to allow for an assessment of the sensitivity of the test method to assess a specific chemical class or product class.

Benchmark substance: A substance used as a standard for comparison to a test substance. A benchmark substance should have the following properties:

- a consistent and reliable source(s)
- structural and functional similarity to the class of substances being tested
- known physical/chemical characteristics
- supporting data on known effects
- known potency in the range of the desired response

Bowman's layer: The anterior lamina of the cornea located under the epithelial layer in some species (e.g., humans, avians, cetaceans) and above the corneal stroma.

Clearing solvent: Substance miscible with ethanol or any other dehydrating agent that is also miscible with an embedding agent such as paraffin wax. Infiltration of this solvent results in clearing of the tissue or in an increase in the transparency of the tissue.

Cornea: The transparent part of the coat of the eyeball that covers the iris and pupil and admits light to the interior.

Corneal opacity: Measurement of the extent of opaqueness of the cornea following exposure to a test substance. Increased corneal opacity is indicative of damage to the cornea. Opacity can be evaluated subjectively as done in the Draize rabbit eye test, or objectively with an instrument such as an “opacitometer.”

Corneal permeability: Quantitative measurement of damage to the corneal epithelium by a determination of the amount of sodium fluorescein dye that passes through all corneal cell layers.

Corneoscleral button: A cornea dissected from an enucleated eye that typically includes a rim of 2-3 mm of scleral tissue.

Corrosive: A substance that causes irreversible tissue damage at the site of contact.

Cutting: Use of a microtome or other knife-bladed instrument to produce thin ribbons of tissue (e.g., 3 to 8 μM for tissue) that can be mounted on glass slides prior to staining.

Davidson's Fixative: A tissue fixative composed of 4% paraformaldehyde in 0.1 M phosphate buffer that may be used in place of 10% neutral buffered formalin (see below) to reduce tissue shrinkage, particularly useful for large ocular tissues (e.g., enucleated globes).

Descemet's membrane: The posterior lamina of the cornea that lies at the posterior end of the stroma and precedes the endothelial layer.

Dehydration: The process of removing the natural water content of the tissue using a series of increasing concentrations of a solvent such as ethanol that is miscible with water

Embedding: Process of surrounding a pathological or histological specimen with a firm and sometimes hard medium such as paraffin, wax, celloidin, or a resin, to allow for cutting thin tissue sections for microscopic examination.

Endothelium: A single layer of flat, hexagonally arranged cells continuous with the iridocorneal angle of the anterior chamber of the eye. The endothelium actively maintains corneal transparency by regulation of fluid exchange with the aqueous humor (Samuelson 2007).

Epithelium: The anterior epithelium covers the anterior corneal surface. It is composed of a thin basement membrane with columnar epithelial cells, followed by two or three layers of polyhedral wing cells, various layers of non-keratinized squamous cells (Samuelson 2007).

Fixation: The process of placing a tissue sample in 5 to 10 volumes of a substance known to stabilize the tissue from decomposition (e.g., 10% NBF or Davidson's fixative) as soon as possible after procurement and trimming. The time needed to infiltrate the tissue depends on the chemical characteristics of the fixative (e.g., ≥24 hr for NBF and no more than 24 hr for Davidson's fixative.

Globally Harmonized System (GHS): A classification system presented by the United Nations that provides (a) a harmonized criteria for classifying substances and mixtures according to their health, environmental and physical hazards, and (b) harmonized hazard communication elements, including requirements for labeling and safety data sheets.

Good Laboratory Practices (GLP): Regulations promulgated by the U.S. Food and Drug Administration and the U.S. Environmental Protection Agency, and principles and procedures adopted by the Organization for Economic Cooperation and Development, and Japanese authorities that describe record keeping and quality assurance procedures for laboratory records that will be the basis for data submissions to national regulatory agencies.

Hazard: The potential for an adverse health or ecological effect. A hazard potential results only if an exposure occurs that leads to the possibility of an adverse effect being manifested.

Histopathology: The science or study dealing with the cytologic and histologic structure of abnormal or diseased tissue.

Infiltration: The passive diffusion of a dehydrating solvent, clearing solvent, or liquid embedding material into a fixed tissue sample.

***In Vitro* Irritancy Score:** An empirically-derived formula used in the BCOP assay whereby the mean opacity and mean permeability values for each treatment group are combined into a single *in vitro* score for each treatment group. The *In Vitro* Irritancy Score = mean opacity value + (15 x mean permeability value).

Limbus: Transition zone between the corneosclera and conjunctiva that houses the collecting vessels for aqueous humor outflow and stem cells for regeneration of epithelium in wound healing.

Negative control: An untreated sample containing all components of a test system, except the test substance solvent, which is replaced with a known nonreactive material, such as water. This sample is processed with test substance-treated samples and other control samples to determine whether the solvent interacts with the test system.

Neutral Buffered Formalin (10%): 10% neutral buffered formalin is a tissue fixative composed of 37 to 40% formaldehyde solution in 0.1 M phosphate buffer, pH 7.4.

Nonirritant: (a) A substance that produces no changes in the eye following application to the anterior surface of the eye. (b) Substances that are not classified as GHS Category 1, 2A, or 2B; or EU R41 or R36 ocular irritants.

Ocular irritant: A substance that produces a reversible change in the eye following application to the anterior surface of the eye.

Opacitometer: An instrument used to measure "corneal opacity" by quantitatively evaluating light transmission through the cornea. The instrument has two compartments, each with its own light source and photocell. One compartment is used for the treated cornea, while the other is used to calibrate and zero the instrument. The difference between photocell signals in the two compartments is measured electronically as a change in voltage, and is displayed digitally, generating numerical opacity values with arbitrary units.

Positive control: A sample containing all components of a test system and treated with a substance known to induce a positive response, which is processed with the test substance-treated and other control samples to demonstrate the sensitivity of each experiment and to allow for an assessment of variability in the conduct of the assay over time.

Sclera: A portion of the fibrous layer forming the outer envelope of the eyeball, except for its anterior sixth, which is the cornea.

Severe irritant: (a) A substance that causes tissue damage in the eye following application to the anterior surface of the eye that is not reversible within 21 days of application or causes serious physical decay of vision. (b) Substances that are classified as GHS Category 1, EPA Category I, or EU R41 ocular irritants.

Solvent control: An untreated sample containing all components of a test system, including the solvent that is processed with the test substance-treated and other control samples to establish the baseline response for the samples treated with the test substance dissolved in the same solvent. When tested with a concurrent negative control, this sample also demonstrates whether the solvent interacts with the test system.

Staining: The addition of substances to tissue that has been processed, cut, and mounted on a glass slide that adds color and permits visualization of the tissue of interest.

Stroma: The framework of connective tissue and keratocytes that provides structure to the eye. The anterior portion of the stroma begins after Bowman's layer or the anterior lamina and ends with Descemet's membrane or the posterior lamina that precedes the endothelial cell layer.

Tissue: A collection of similar cells and the intercellular substances surrounding them. There are four basic tissues in the body: 1) epithelium; 2) connective tissues, including blood, bone, and cartilage; 3) muscle tissue; and 4) nerve tissue.

Tissue processing: The protocol followed for fixation, post-fixation trimming, dehydration, clearing, and embedding of tissue for use in histology.

Trimming: The process of removing non-critical, excess tissue before or after fixation by cutting with scissors or a scalpel to minimize a tissue sample to those sections that are needed for the evaluation.

Validated test method: An accepted test method for which validation studies have been completed to determine the relevance and reliability of this method for a specific proposed use.

Weight of evidence (process): The strengths and weaknesses of a collection of information are used as the basis for a conclusion that may not be evident from the individual data.

ANNEX II

FORMULATIONS*

Fixatives

10% Neutral Buffered Formalin (NBF)

Formaldehyde, 40%	10 mL
Distilled water	90 mL
Sodium dihydrogen phosphate (NaH_2PO4)	0.35 g
Disodium hydrogen phosphate (Na2HPO4) (anhydrous)	0.65 g

Paraformaldehyde

Dissolve paraformaldehyde in distilled water to make a 40% solution by heating in a fume hood, stirring constantly until only a faint cloudiness persists. Clarify this solution by adding 10N NaOH drop by drop until the solution is clear. Cool to room temperature and then to 4°C in a refrigerator. Filter after cooling to 4°C. If the solution is being used only for subsequent paraffin sections, the longevity of the 40% paraformaldehyde can be increased by adding sodium pyrophosphate in the 40% paraformaldehyde solution.

6% Glutaraldehyde

Glutaraldehyde (25%)	24 mL
0.1 M Phosphate buffer, pH 7.4	76 mL

On storage, the glutaraldehyde will become acidic, pH 2.5-3.0. The final pH of the above fixative should be checked and adjusted to 7.0-7.2 if necessary with sodium hydroxide.

Davidson's Fixative†

Formalin (37-40%)	40 mL
Alcohol	60 mL
Glacial acetic acid	20 mL
Tap water	60 mL

Mix before using, immerse for less than 24 hr and transfer to 70% alcohol prior to embedding.

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†(18)

773 ***Buffers***
774
775 **Phosphate buffer (Sorenson's), pH, 7.4**[‡]
776
777 0.1 M Sodium dihydrogen orthophosphate stock (NaH_2PO_4); MW 156
778 • Sodium dihydrogen orthophosphate 1.56 g
779 • Distilled water 100 mL
780
781 0.1 M Disodium hydrogen orthophosphate stock (Na_2HPO_4); MW 142
782 • Disodium hydrogen orthophosphate stock 1.415 g
783 • Distilled water 100 mL
784
785 0.1 M Phosphate Buffer, pH 7.4
786 • 9 mL NaH_2PO_4 and 41 mL of Na_2HPO_4 at 25°C
787

[‡](38)

ANNEX III

SAMPLE PROTOCOLS

Sample Protocol #1 for Routine Hematoxylin and Eosin Staining of Paraffin-Embedded Sections[§]

1. Deparaffinize sections; hydrate through graded alcohols to water.

2. Remove fixation pigments (i.e., from bloody deposits or acidic formalin, but not a concern if NBF is used).

3. Stain in a alum hematoxylin of choice for a suitable time (e.g., 20-45 min for Ehrlich's)

4. Wash well in running tap water until sections 'blue' for 5 min or less.

5. Differentiate in 1% acid alcohol (1% HCl in 70% alcohol) for 5-10 sec.

6. Wash well in tap water until sections are again 'blue' (10-15 min).

7. Blue by dipping in an alkaline solution (e.g., ammonia water), followed by a 5-min tap water rinse.

8. Stain in 1% eosin Y for 10 min.

9. Wash in running tap water for 1-5 min.

10. Dehydrate through alcohols, clear, and mount.

Sample Protocol #2 for Routine Hematoxylin and Eosin Staining of Paraffin-Embedded-Sections[**]

1. Hydrate the tissue:

   - Fix a microscope slide holding rehydrated tissue sections in either alcohol or an aldehyde-based fixative.
   - Immerse the slide in water for 30 sec with gentle agitation by hand to prevent precipitation with salts or buffers.

2. Dip the slide into a Coplin jar containing Mayer's hematoxylin and agitate for 30 sec.

3. Rinse the slide in water for 1 min.

[§]Modified from (32)
[**]Modified from (26)

4. Stain the slide with 1% eosin Y solution for 10-30 sec with agitation.

5. Dehydrate the sections with two changes of 95% alcohol and two changes of 100% alcohol for 30 sec each.

6. Extract the alcohol with two changes of xylene to clear.

7. Add one or two drops of mounting medium and cover with a cover slip. Glycerol can be used if alcohols cannot be used.